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UNSATURATED TRIGLYCERIDE AND FATTY ACID LIPOXIDASE ACTIVITIES OF NAVY BEANS, SMALL RED BEANS, PEANUTS, GREEN PEAS AND LIMA BEANS [a]

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Siddiqi and Tappel *(6)* have reported the presence of lipoxidase activity in a number of legumes using linoleate salts as substrates. Earlier these same workers investigating pea lipoxidase *(5)* suggested an implication of lipoxidase action to the production of off odors in underblanched frozen peas. Wagenknecht and Lee *(9)* observed a similar relationship and postulated that the pea lipase produced the substrate for lipoxidase action. However, Koch *et al (3)* found relatively high peroxide values and corresponding "off odors" were produced when navy bean lipoxidase acted on cottonseed oil without a noticeable increase in free fatty acid.

Koch *et al (2)* recently reported that soybeans are capable of oxidizing by apparent lipoxidase action both linoleic acid and the tri-glyceride trilinolein. Presumptive evidence was presented for the existence of two separate enzymes, one which can act on natural triglycerides and which may be responsible for "off odor" development in natural products containing little free fatty acids.

This paper reports further studies on the existence of the two types of lipoxidase activity in other legumes. Also presented are studies on the prevention of off odor development in enzyme inactivated products.

MATERIALS AND METHODS

Enzyme extraction. Dried raw navy beans, peas, small red beans, lima beans, and peanuts ground in a Wiley mill to pass through a No. 20 sieve, and soy flour were used as enzyme sources. The soy flour and ground peanuts were each defatted with several changes of Skellysolve F and solvent was evaporated before use. The ground legumes were extracted with distilled water (5 g per 50 ml) by stirring to keep particles in suspension for 10 min and then insolubles were removed by centrifugation. Calcium chloride (64 mg per ml of extract) was added to the supernatant to precipitate inactive protein. The precipitate was removed by centrifugation and the supernatant further clarified by filtration through Whatman No. 4 filter paper. The filtrates were stored at 4° C until used. Except where specifically stated, extracts were made fresh daily.

Substrates. Substrates used were highly purified trilinolein and linoleic acid obtained from The Hormel Institute, molecularly distilled cottonseed oil (to remove tocopherols), and wheat flour oil (extracted from patent flour with Skellysolve F). Triglyceride substrates were prepared by dissolving sufficient oil in a 50:50 mixture of acetone and 95% ethanol to give 20 mg oil per ml of solution. Presence of the ethanol prevented autoxidation of the oils. Linoleic acid was dissolved in 95% ethanol at a concentration of 10 mg per ml of solution.

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Reaction mixture. The reaction mixture consisted of 100 ml distilled water, 5.0 ml buffer solution (0.2M), 1 ml substrate solution, and appropriate amount of enzyme extract to give suitable hydroperoxide formation in the desired time interval. Reaction temperature was 20° C.

Lipoxidase assay. The method described by Koch *et al* was followed for measurement of lipoxidase activity. This included hydroperoxide determination by the thiocyanate method of Sumner *(7)*.

RESULTS AND DISCUSSION

Navy beans, peanuts, small red beans, green peas and lima beans were investigated following the same procedures as those reported for soybeans *(2)*. All of these products showed differences in lipoxidase activity characteristics similar to soybeans with some variations as noted below.

pH studies. The effect of pH on lipoxidase activity in navy beans was studied using 4 different substrates, Figure 1.

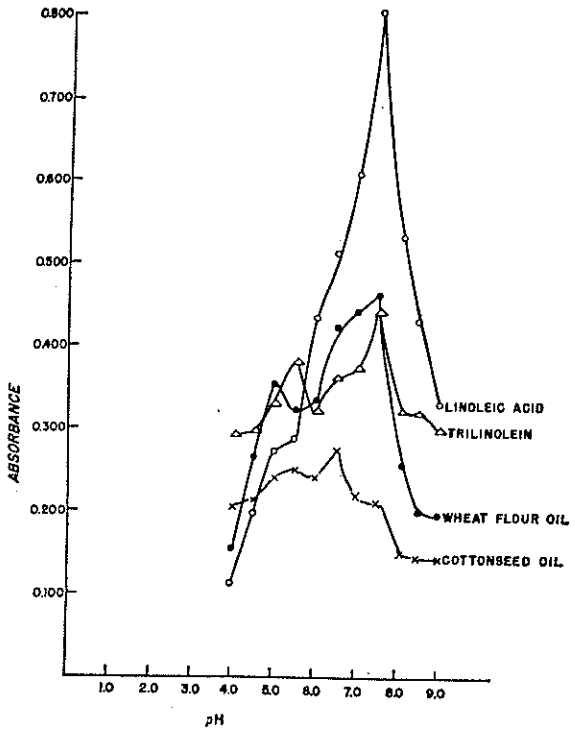

Figure 1. Effect of pH on activity of navy bean lipoxidase for 4 minute reaction time. Reaction mixture: 3.5×10^{-4} M linoleic acid or 2.2×10^{-4} M trilinolein, cottonseed oil, and wheat flour oil, 0.5 ml extract (100 mg/ml), 20° C.

When linoleic acid was used as the substrate, only one sharp peak was observed. Optimum activity occurred at pH 7.5, slightly lower than the pH 8.3 optimum for soybeans; this activity of navy beans decreased more rapidly than that of soybeans above the pH optimum. pH activity curves were also obtained on 3 different triglyceride substrates. The triglyceride active material showed two apparent pH optima for each of the triglycerides.

Using trilinolein, peaks occurred at pH 7.5 and 5.5 with the greater activity at the higher pH optimum, which is the opposite of that observed for soybeans *(2)*. The rate of lipoxidase action was greater on wheat flour oil than on trilinolein at the higher pH optimum despite the fact that flour oil has only about 50% linoleic acid. The lower pH optimum with flour oil was 5.0. Cottonseed oil, which also contains approximately 50% linoleic acid, was the poorest substrate and showed only one definite peak at pH 6.5. These results on the different triglycerides indicate that the triglyceride active material possesses a substrate specificity.

The effect of pH on the lipoxidase activity of peanuts is shown in Figure 2 for three different substrates. With linoleic acid as substrate, a sharp peak occurred at pH 8.1 (similar to soybeans) and a slight hump at pH 6.0. In this product rates of enzyme action on the fatty acid and triglyceride substrates were not so widely separated as in navy beans and soybeans. Two pH optima were observed for each of the two triglycerides tested. Optimum activity occurred at pH 7.5 and 5.5 for the trilinolein substrate with a slightly greater rate of action at the lower pH which is similar to soybeans, but the opposite of navy beans. Cottonseed oil was a good substrate for peanut lipoxidase. Its rate of activity at pH 7.5 was greater on cottonseed oil than on trilinolein. A second peak in the pH curve with cottonseed oil was noted at pH 6.5. This difference in rates of activity on the two triglyceride substrates again indicates a substrate specificity by the legume lipoxidases.

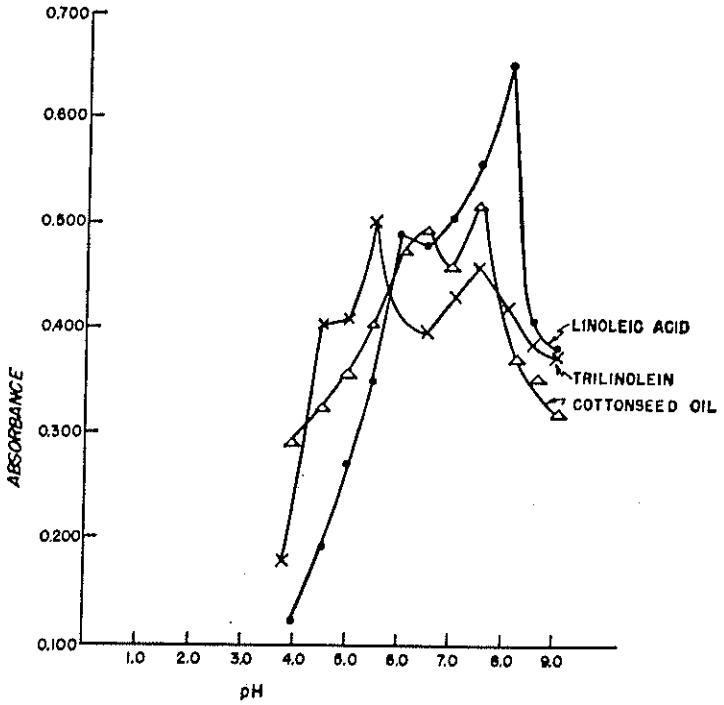

Figure 2. Effect of pH on activity of defatted peanut lipoxidase for 4 minute reaction time. Reaction mixture: 3.5 x 10^{-4} M linoleic acid or 2.2 x 10^{-4} M trilinolein and cottonseed oil, 0.5 ml extract (100 mg/ml), 20° C.

Cottonseed oil was a good substrate for peanut triglyceride active enzyme; it was a poor substrate for the navy bean triglyceride active enzyme.

Figure 2 shows that defatted peanuts (raw Spanish Red variety) contained a substantial amount of lipoxidase activity. Comparing the quantity of extract to give the same absorbance values in a 4-min reaction time, peanuts had approximately 40% of the trilinolein activity of soybeans at pH 5.5. However, peanuts had only 10% of the linoleic acid activity of soybeans at pH 8.1. These values are considerably higher than that reported by Siddiqi and Tappel *(6)* who used pH 7.0 for their assay and found that peanuts on a linoleate substrate had only 1% of the activity of soybeans. Although peanuts are rather poor in linoleic acid activity, next to soybeans they possess the most active trilinolein lipoxidase activity of the 5 legumes reported, Figure 6.

Green pea lipoxidase activity was tested on only two substrates (Figure 3). This product had less total activity than the previous two legumes, but again showed the same two peaks in the pH activity curve with trilinolein as the substrate, i.e., 5.5 and 7.5. Maximum activity on linoleic acid occurred at pH 7.5, the same pH as in navy beans. This is in close agreement with Tappel *et al (8)* who used 0_2 absorption to measure lipoxidase activity on a linoleate substrate. They reported the optimum to be pH 6.9. They did not test for triglyceride activity.

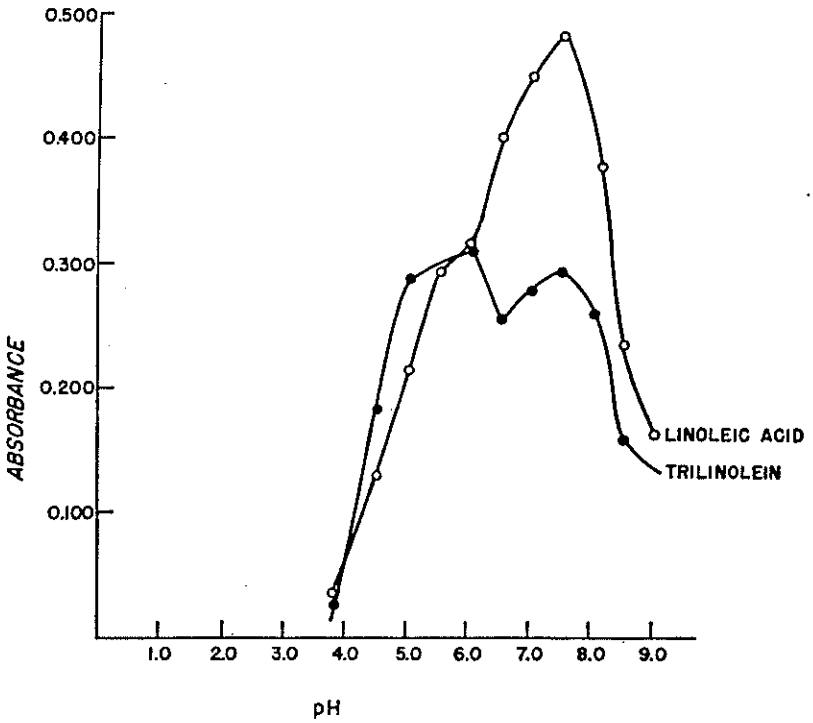

Figure 3. Effect of pH on activity of green pea lipoxidase for 4 minute reaction time. Reaction mixture: 3.5×10^{-4} M linoleic acid or 2.2×10^{-4} trilinolein, 0.5 ml extract (100 mg/ml), 20° C.

Small red beans had a wide difference in the two types of activity, possessing a much greater lipoxidase activity on linoleic acid, Figure 4. Hydroperoxide production from linoleic acid was greatest at pH 7.0, while using trilinolein optima occurred at pH 5.5 and 7.5.

Baby lima beans and large lima beans were the last of the legumes tested. They contained about equal amounts of lipoxidase activity and contained the lowest level of activity yet observed. The pH activity curves for baby lima beans using linoleic acid and trilinolein as substrates are shown in Figure 5. Activity on the trilinolein substrates was very low and showed a broad range of maximum activity between pH 5.5 and 7.0. However, the trilinolein active material was quite labile in the extract and showed a rapid loss in activity which made it difficult to obtain reproducible results.

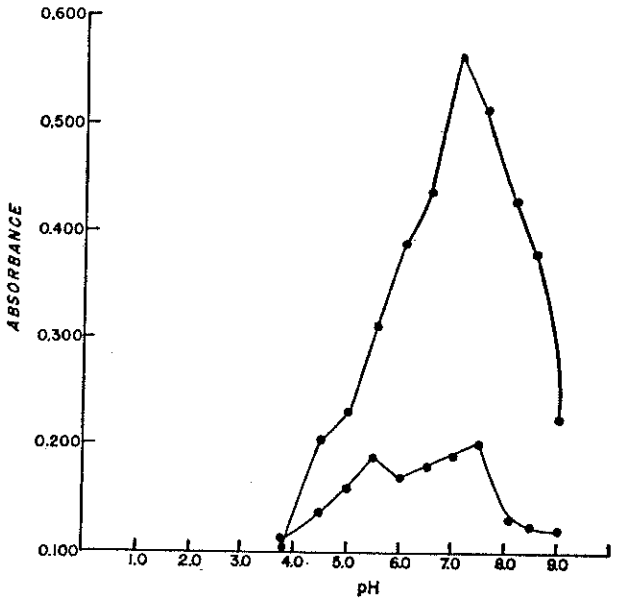

Figure 4. Effect of pH on activity of small red bean lipoxidase for 4 minute reaction time. Reaction mixture: 3.5 x 10^{-4} M linoleic acid or 2.2 x 10^{-4} M trilinolein, 0.5 ml extract (100 mg/ml), 20° C.

It may be possible that this product also has the double triglyceride peak observed in the other legumes.

The enzyme active on linoleic acid in lima beans was more stable and had a sharp optimum at pH 7.5. But it was necessary to use 1 ml of extract, compared to 0.5 ml of extract for the other 4 legumes reported, in order to obtain sufficient hydroperoxide production in a 4-minute reaction time.

Holding the ground lima beans overnight at −4° C did not prevent loss of trilinolein activity (Table 1). It can also be seen, Table 1, that there was a long lag period when 0.5 ml extract was added to a reaction mixture with linoleic acid. When 0.2 ml extract was added, no activity was noted in 16 min. The lag period was no longer apparent when 1 ml of extract was used.

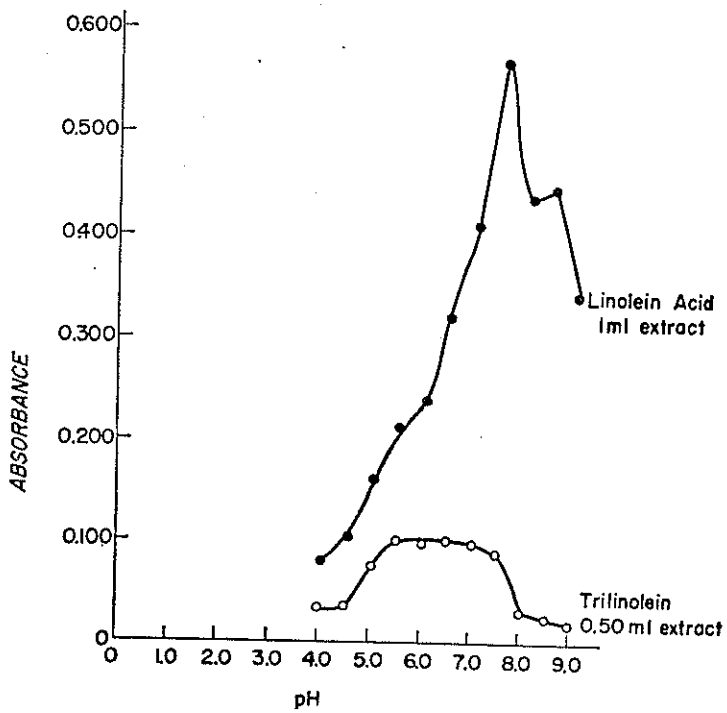

Figure 5. pH activity for baby lima beans, linoleic acid and trilinolein as substrates.

Time studies. Time studies were also carried out on the other 4 legumes. Results are presented in Figures 6 and 7. Using a trilinolein substrate the time course of hydroperoxide production was similar for each legume. However, there was an apparent difference in enzyme content as shown by amount of activity at any given time, Figure 6. Except for peanuts the time curves obtained with a linoleic acid substrate were also similar for the different legumes, but were different from those on trilinolein, Figure 7. A lag period occurred like that reported for soybeans *(2)*. Peanut linoleic acid lipoxidase activity did not show a lag period and followed a time course of reaction similar to the trilinolein active enzyme.

Note that the apparent content of lipoxidase enzyme for trilinolein and linoleic acid shows a considerable variation in the 4 legumes shown in

TABLE 1

Time course of lipoxidase activity of ground lima beans stored overnight at 4° C[1]

Absorbance

Time	Substrate Trilinolein (pH 5.5)	Linoleic Acid (pH 7.5)	
(Min)	*(1.0 ml Ext)*[2]	*(0.50 ml Ext)*[2]	*(1.0 ml Ext)*[2]
2	0.007	0.000	0.152
4	0.004	0.032	0.332
8	0.010	0.164	0.712
16	0.022	0.463	0.760

[1] Reaction mixture: 2.2×10^{-4} M trilinolein or 3.5×10^{-4} M linoleic acid, 0.01 M buffer, 20° C.
[2] Extract concentration: 100 mg/ml.

Figures 6 and 7. Navy beans, which had the highest linoleic acid activity, were third in amount of trilinolein activity. Amount of peanut and green pea lipoxidase activity also shifts positions on the two substrates. The above, together with substrate pH optima differences, would seem to favor the possibility of two different types lipoxidase enzyme and supports similar evidence presented for soybeans *(2)*.

It was also noted that one of the double peaks obtained with the trilinolein substrate occurred at the same pH as the optimum pH for linoleic acid activity for all of the 5 legumes under investigation. This was also observed for soybeans *(2)*. However, for soybeans it was shown that the linoleate active enzyme has little or no activity on the triglyceride substrate. This is also believed to be true for the other legumes. Time studies on the 2 substrates at low enzyme levels of baby lima beans showed distinct differences, Figure 8. A definite induction period occurred for the linoleic acid activity; no lag was observed for equally low trilinolein activity.

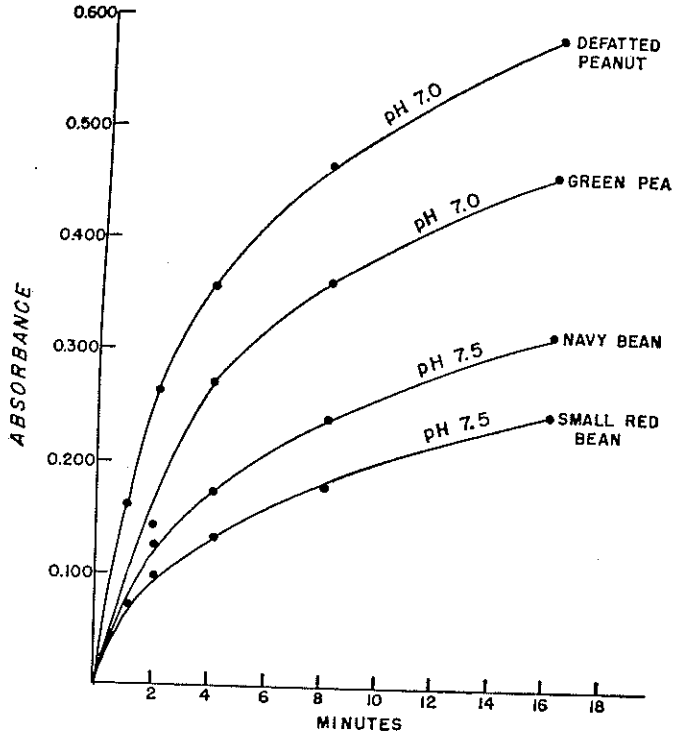

Figure 6. Time curve for 4 legume extracts. Reaction mixture: 2.2×10^{-4} M trilinolein, 0.2 ml extract (100 mg/ml) 20° C.

The reason for the induction period for linoleate active lipoxidase observed with several of the legumes is unknown. Haining and Axelrod *(1)* have reported on the induction period for soybean lipoxidase on a linoleate substrate. They found that minute amounts of hydroperoxide could eliminate the lag in the enzyme oxidation and concluded that hydroperoxide acts as an enzyme activator. An equally plausible explanation of their findings

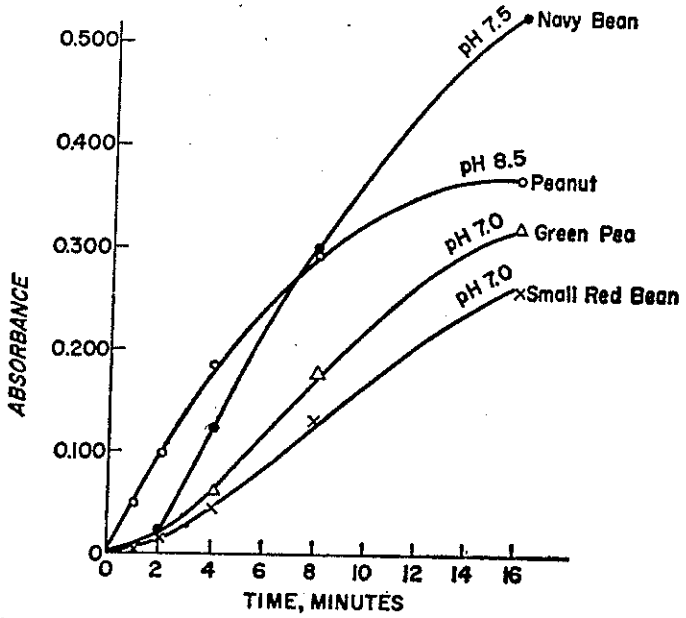

Figure 7. Time studies, linoleic acid substrate, for four legumes.

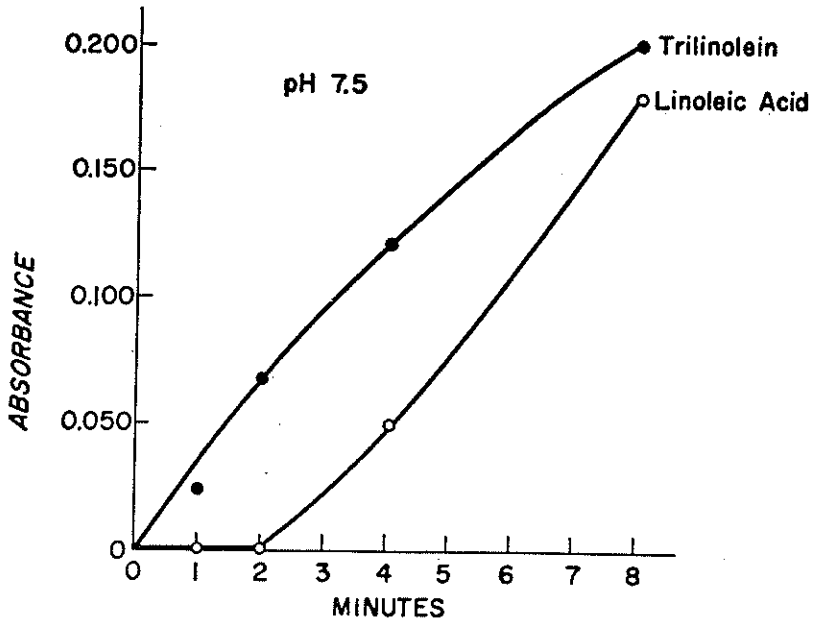

Figure 8. Time studies at low enzyme levels, baby lima beans.

TABLE 2

The effect of hydroperoxides on the induction period in linoleate lipoxidase reactions

A. Baby Lima Beans

	Absorbance		
Initial	0.000	0.051	0.143
(min)	*Increase over initial*		
2	0.000	0.000	0.002
4	0.000	0.000	0.060
8	0.012	0.020	0.107
16	0.097	0.099	0.284

Reaction mixture: 3.5×10^{-4} M linoleic acid, 0.20 ml extract (100 mg/ml), 0.01 M buffer pH 7.5, 20° C.

B. Soybeans

	Absorbance		
Initial	0.000	0.310	0.510
(min)	*Increase over initial*		
1	0.000	0.000	0.015
2	0.000	0.000	0.085
4	0.009	0.052	0.065
8	0.110	0.172	0.235
16	0.319	0.322	0.405

Reaction mixture: 3.5×10^{-4} M linoleic acid, 0.20 ml extract (50 mg/ml), 0.01 M buffer pH 8.3, 20° C.

might be that the hydroperoxide inactivates natural inhibitors present in the enzyme extract. Privett *et al (4)* have shown that the linoleic acid lipoxidase of soybeans is specific for a cis-cis 1,4-pentadiene system, and that conjugated diene systems inhibit the enzyme. It is difficult therefore to see how the linoleate hydroperoxide, which has a conjugated diene structure, can also be an enzyme activator.

In an effort to check on the effect of hydroperoxide on the lipoxidase induction period of baby lima bean and of soybean extracts, time studies were conducted at several initial peroxide levels, Table 2. It appears that a higher peroxide content is required to cause a noticeable decrease in the induction period for soybeans than for lima beans. Although these data do not resolve unequivocally the question of enzyme activation or inhibitor inactivation by hydroperoxide, the very much higher requirement of the soybean extract for equivalent reduction of the induction period, makes the inhibitor inactivation hypothesis more attractive. Soybeans, which contain more lipoxidase activity than lima beans, may also contain more inhibitors of this activity.

Comparison of the hydroperoxide required to shorten the induction period reported here with that reported by Haining *(1)* may be of interest. Standardization of the ferric thiocyanate colorimetric method for determination of peroxide showed than an absorbance of 0.100 is equivalent to 4.5 mc moles of linoleate hydroperoxide in the enzyme reaction mixture. Thus from Table 2, significant reduction of the induction period required 6 mc moles for lima beans and 14 mc moles for soybeans. Haining and Axelrod *(1)* reported a decrease of one-half in the induction period with 1×10^{-3} mc mole of linoleate peroxide and its abolition by 5×10^{-3} mc mole.

One further observation may be made from Table 2. The soybean system whose initial absorbance was 0.510 contained 40 ml of an oxidation mixture containing 35 mc mole of linoleic acid. The peroxide content of this oxidation mixture was 57.5 mc mole, indicating that nearly two-thirds of the original linoleate had been oxidized to a di-peroxide. In other experiments, using small substrate concentrations and oxidizing for long periods, more than 95% of the original linoleate apparently had been carried to diperoxide.

Enzyme stability in extract solutions. As reported above, Table 1, the triglyceride activity in lima beans was quite labile after the whole beans were ground. The other legumes also showed a gradual loss of lipoxidase activity with time after grinding. However, the stability of the enzymes in the extract solutions were the opposite from lima beans. Navy beans, small red beans, and green peas were not as readily inactivated and the linoleic acid activity showed the greatest decrease with time. Peanuts showed the most rapid loss of linoleic acid activity with time, while its triglyceride activity was quite stable when held in solution at 4° C. Thus there seems to be a variation between different legumes in the stability of the two types of lipoxidase activity which adds further evidence for the possible existence of two distinct types of lipoxidase.

SUMMARY

Lipoxidase activities of crude extracts of five different legumes were studied using triglyceride and linoleic acid as substrates.

Variations in pH optima were noted for the different substrates. The triglyceride activity showed two distinct peaks in the pH curves and an apparent substrate specificity for different triglycerides.

Using low level of enzyme activity a lag or induction period was observed with linoleic acid as the substrate for all but one legume tested, while no lag was observed with the trilinolein substrate.

The lipoxidase activity for the two types of substrates also showed variations in stability in the legume extracts.

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